STUDIES ON COMPLEMENT.

A Thesis

for the degree of Doctor of Medicine 1921.

by

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I N T R O D U C T I O N.

Many people still believe that complement is a substance present in fresh serum though not yet isolated. Nobody however will deny that complement is a property of fresh serum. No true substitute for complement in the haemolytic system has been found. Noguchi (1907) made certain "artificial complements" out of soaps but could not make them carry out every test. Stronger haemolysis can be produced by adding soaps to normal sera (Von Liebermann and von Fenny-vessy 1911 and 1912), and in the case of certain sera by adding certain lecithins (Cruickshank and Mackie 1913 and 1914). In these experiments no substance was found capable of replacing complement.

In some ways complement resembles an enzyme, but it seems to participate in reactions according to the law of definite proportions (Von Liebermann 1906).

A great deal of work has been done on the split products of complement but when the serum is split the complement is split, and it is not proved that complement is present in only one fraction. (Eastwood 1920. I).

Ritchie and McGowan (1912) recorded an interesting fact/

fact obtained by alternately freezing and thawing serum in a long burette. They found that while the nitrogen and ash were concentrated about six times in the bottom of the tube as compared with the top, the complementing power is concentrated thirty-three times at least. This experiment does not prove that complementing power and serum protein are identical, for the former is concentrated out of all proportion to the latter.

The extremely complicated nature of complement is brought out by the following two references.

Browning and Mackie (1914) were able by dialysis to reduce the deviability of complement greatly without effecting practically any alteration in its haemolytic activity.

Griffith and Scott (1920) working with guinea pig serum showed that haemolytic activity, sensitiveness to antigen, and specific fixability are not related. It was found impossible to tell from the degree of any one of these functions the degree of any other.

Eastwood(1920. 2) summarizing recent work on complement says:- "The purely chemical and quantitative conception of the activities of complement, whether presented in a simple or in a complicated form/

form has failed and must be replaced by something else."

The work now to be described is divided into two parts:-

1. Experiments on the relation of certain healthy and diseased conditions to the haemolytic activity of the serum.
2. Experiments on the nature of complement.

Only haemolytic complement was studied throughout.

I wish to record my thanks to Professor Ritchie for his advice and encouragement, and to the Carnegie Trustees and to the Trustees of the Moray Endowment for financial assistance.

T E C H N I Q U E.

Throughout the experiments the following methods were employed.

Serum - Complement:- Guinea pigs were bled by cutting their throats, rabbits by incising an auricular vein. The blood was allowed to stand at room temperature for one hour and then the clot was loosened and the specimen put in the ice-chest. Next day (21 - 26 hours after bleeding) the serum was pipetted off and centrifuged clear of cells.

Immune body:- Rabbit serum v. sheep cells was tested out every 4 - 6 weeks in excess of complement. The haemolytic titre was found to decrease very slowly.

Sheep Cells were collected and defibrinated at the slaughter house and were washed the same day three times in saline. Cells were occasionally required when they could not be obtained fresh, and they were then used after being treated with formalin (0.1 cc. of 1 in 10 dilution for every 5 cc. of sediment) as recommended by Armand - Delille and Launoy (1911). This method was found satisfactory.

The tests were always carried out on a plan similar to the following table. The quantities are in fractions of a cc.

TABLE I./

TABLE I.

Tube number.	1	2	3	4	5	6
Guinea pig serum diluted 1 in 20.	0.3	0.25	0.2	0.15	0.1	0.05
Saline 0.85 per cent.	0.4	0.45	0.5	0.55	0.6	0.65
Immune body 1 in 300 (M.H.D. 0.1 of 1 in 300)	0.3	0.3	0.3	0.3	0.3	0.3
Sheep Cells 1 per cent.	1.0	1.0	1.0	1.0	1.0	1.0

The tubes were thoroughly shaken and then incubated at 37° C. for one hour during which they were shaken at least twice. After one hour the reading was taken and the lowest tube completely free from cells was noted as giving the minimum haemolytic dose (M.H.D.). This has been always recorded as a fraction of a cc. of neat serum.

Inactivated serum was obtained by heating fresh serum at 55° C. for ten minutes as recommended by Harrison (1918). On several occasions serum heated in this way was tested and no haemolysis was found even in 0.7 of neat serum. The serum was not heated for longer than ten minutes at 55°C. because the destruction of complement was desired with the minimum of chemical or physical change in the serum.

PART I.

SURVIVAL OF ACTIVITY OF COMPLEMENT.

In the first weeks of this work serum was tested for survival of complement. Complement survived longer than was anticipated and remained constant for three or four days when kept in the ice chest. The case recorded with Table II is noteworthy.

Experiments:- Guinea pig killed 12 noon 8/6/20. After 23 hours in ice chest serum was pipetted off and centrifuged. Serum was then divided into two parts.- 1. kept in ice chest. 2. at room temperature.

TABLE II.

Hours since bled.	24	48	72	96	120	144	168	192	216	240
M.H.D. No.1.	0.005	0.005	0.01	0.01	-	0.01	0.03	0.06	?0.3	?0.6
M.H.D. No.2.	"	"	"	0.05	-	-	-	-	-	-

In seven separate specimens of rabbits serum no appreciable difference was found in amount of complement up to 72 hours. The serum was tested after 24, 48, 72 hours in ice chest.

On testing serum 1 - 4 hours old the M.H.D. was generally irregular, sometimes smaller but usually larger than that of serum 24 hours old. This point has been noted several times and was specially emphasized by Gurd (1912).

COMPLEMENT IN HEALTH AND DISEASE.

In healthy guinea pigs many people have recorded very slight variations in complementary activity (Gurd 1912, Dick 1913, Griffith and Scott 1920). Griffith and Scott note specially the importance of avoiding pregnant animals, if a constant strength of complement is wanted. Gunn (1914) used human blood serum and noted only slight variations in strength when he tested every few hours during two days.

Experiments:- (1) One rabbit (B_1) was tested on nine separate occasions during two months and the M.H.D. varied from 0.04 to 0.08.

(2) One rabbit (W_1) was tested on two occasions with interval of $1\frac{1}{2}$ months and M.H.D. was 0.04 and 0.06.

During disease variations in complement content of the blood have been recorded by Dick (1913). He gives numerous references to literature on the subject and an extensive series of experiments, and concludes that there is a parallelism between liver destruction and complement diminution.

The following experiments were carried out on animals which chanced to be diseased during the course of other laboratory work.

Experiments:-

1. Rabbit (B_1) four days after non-lethal dose of pneumococci (which caused loss of weight only) gave M.H.D. 0.06.
   Rabbit (B_2) three days after dose of pneumococci (which caused death within three days of taking the specimen of blood) gave M.H.D. 0.1.
   Rabbit (W_1) three days after dose of pneumococci (which caused death within 24 hours of taking specimen of blood) gave M.H.D. 0.06.

2. Rabbit (C.) bled immediately after sudden death from shock from overdistended stomach gave M.H.D. 0.08.
   Rabbit (H.) under similar conditions gave M.H.D. 0.075.

3. Three separate guinea pigs killed while very weak and in bad condition (apparently due to deficiency of green food) gave M.H.D. 0.01.

4. One guinea pig (considerable wasting, poor condition, tuberculous inguinal gland, and some minute tuberculous foci in spleen) gave M.H.D. 0.0125.

One/

One guinea pig (tuberculous inguinal and lumbar glands, spleen clear) gave M.H.D. 0.0075.

5. One guinea pig (very many active foci of Pseudo-tuberculosis in liver and a few in spleen, some wasting) gave M.H.D. 0.005.

Conclusions:- From this record of experiments it is interesting to note how seldom there was much alteration of complement activity. Most of the cases recorded were grossly diseased and yet the M.H.D. varied only slightly. In two cases of (non-protein) shock (No.2) there was no loss of complement, while in anaphylactic shock great destruction of complement is recorded by Scott (1911). In advanced cases of faulty nutrition (No.3) the M.H.D. was only slightly altered. On the other hand animals subjected to low temperatures have sera with markedly lowered haemolytic power. This was found to be a great handicap in an Army Laboratory where guinea pigs could not be kept sufficiently warm during the winter. In No.5 the liver tissue was largely destroyed by foci of disease and the M.H.D. was about normal. This result is contrary to the cases of liver destruction with loss of complement recorded by Ehrlich & Morgenroth(1901) & Dick (1913).

VARIATIONS IN COMPLEMENT CONTENT OF SERUM DURING IMMUNISATION.

Gunn (1914) after working with cases of Typhoid concluded that complement is as a rule more abundant during pyrexia than during convalescence, being increased in amount during relapses and diminished when recovery is taking place. He found that complement and immune body are not produced in any fixed ratio to one another. Gunn tested cases of erysipelas and thinks that "irregularity in the amount of complement present during illness seems to indicate a certain degree of instability in the immunising mechanism, as is shown by the clinical course of a case of erysipelas migrans".

Loncope (1903) worked with bacteriolytic complement and found that serum from three cases of Typhoid showed a decrease in bacteriolytic power for b. Typhosus and b. Typhosus alone. He thinks that complement may be looked on as an index to the resistance of the patient.

Walker (1903) found that bacteriolytic power of the sera of immunised animals was markedly greater than that of normal sera except in the case of animals killed/

killed while in a condition of pyrexia and impaired nutrition following inoculation. In them the bacteriolytic action of the serum was very definitely less than that of normal animals.

From the authors it seems evident that increase of bacteriolytic and decrease of haemolytic complement are brought about by similar conditions.

Experiment:- Two rabbits were tested weekly over a period of two months. The presence of complement and agglutinin were estimated in the sera. One rabbit was immunised with b.typhosus, and the other with b. dysenteriae (Flexner). The latter strain was capable of killing another similar rabbit in dose of $\frac{1}{10}$ living agar slope culture intravenously. Rabbit A was ill and off food for two days after first intravenous injection, but soon recovered and otherwise was in good health throughout experiment. Rabbit B. continued in good health throughout. The rabbits were weighed each week and the variations were slight. Agglutination was carried out by macroscopic method allowing two hours at 55°C. and standing for $\frac{1}{2}$ hour at room temperature before reading with a lens. Emulsions from 24 hour agar cultures were used.

TABLE III.

Dates on which bled.		11/10/20	18/10/20	25/10/20	1/11/20	8/11/20	15/11/20
Rabbit A.	M.H.D. Agglutination of B. Typhosus.	0.05 0 in 1/10	0.05 1/200	0.06 1/600	0.08 1/1800	? 0.4 1/10,000	? 0.08 1/7500
Rabbit B.	M.H.D. Agglutination of B. Flexner.	0.06 0 in 1/10	0.05 1/200	0.07 1/2000	0.05 1/4000	0.07 1/4000	0.05 1/2000
Dose of B.Typhosus or B.Flexner and date given.		12/10/20. Dead Subcutaneously. 1/5	19/10/20 ½ ditto	26/10/20 1 ditto.	2/11/20 2 ditto.	9/11/20. 1 living Subcutaneously 1/5	16/11/20 1 ditto.

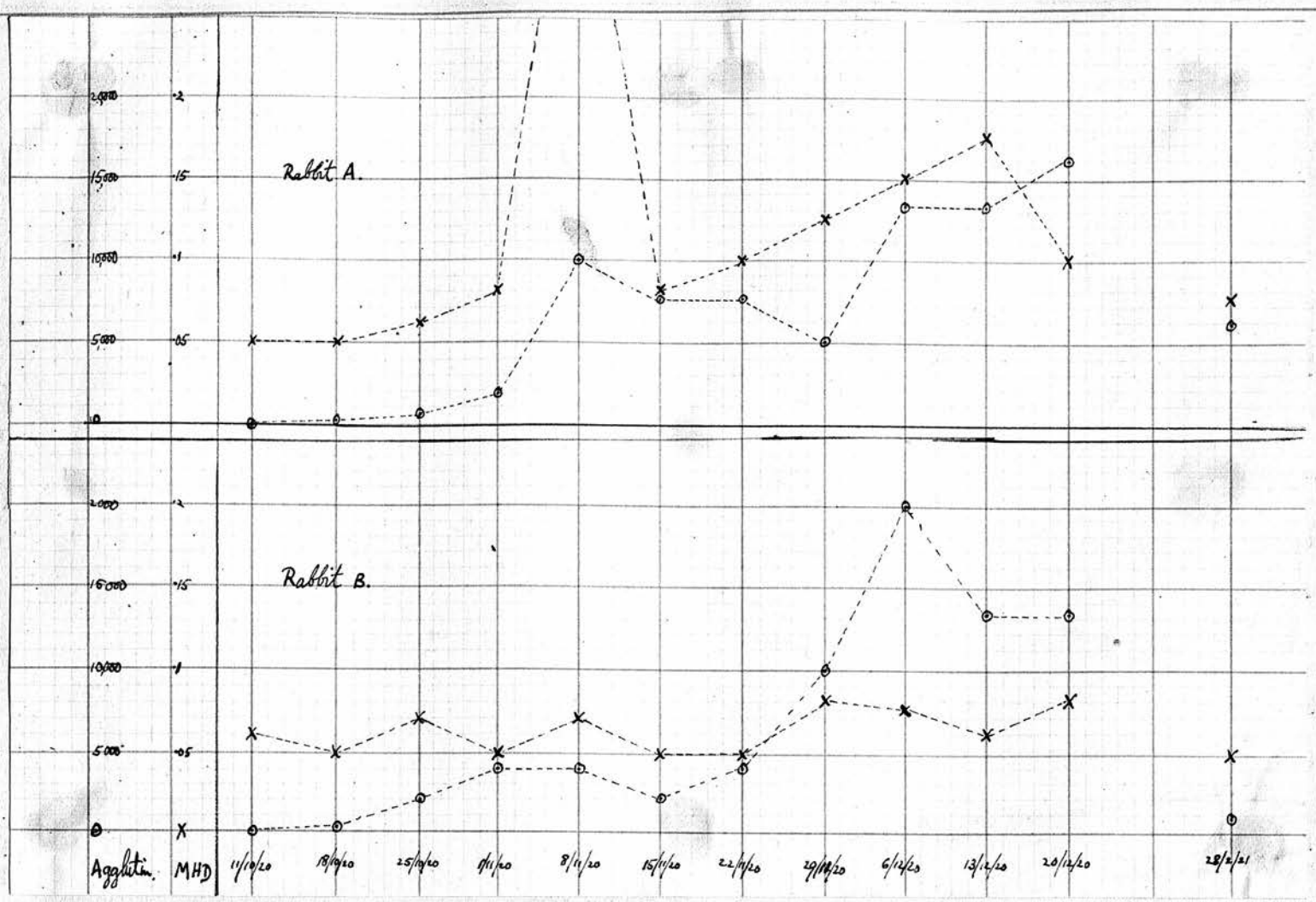
Rabbit A.
Rabbit B.
Agglutin.
MHD
11/10/20
18/10/20
25/10/20
1/11/20
8/11/20
15/11/20
22/11/20
29/11/20
6/12/20
13/12/20
20/12/20
28/2/21

TABLE III. (Cont^{d}.)

Dates on which bled.		22/11/20	29/11/20	6/12/20	13/12/20	20/12/20	28/2/21.
Rabbit A.	M.H.D.	0.1	0.125	0.15	0.175	0.1	0.075
	Agglutination of B. Typhosus.	1/7500	1/5000	1/13,333	1/13,333	1/16,000	1/7500
Rabbit B.	M.H.D.	0.05	0.08	0.075	0.06	0.08	0.05
	Agglutination of B. Flexner.	1/4000	1/10,000	1/20,000	1/13,333	1/13,333	1/750

Dose of B.Typhosus or B.Flexner and date given.

23/11/20. $\frac{1}{8}$ living intravenously

30/11/20 $\frac{1}{8}$ ditto.

7/12/20 $\frac{1}{3}$ ditto.

14/12/20 1 ditto.

Table III shows the details of the experiment. The doses of organisms administered are given in fractions of saline emulsion from 24 hour culture on sloped agar tube. The first four doses consist of organisms killed by heating at 60°C. for one hour and given subcutaneously. The last four doses consist of living organisms given intravenously.
The readings of M.H.D. (Rabbit A) on 8/11/20 and 15/11/20 are approximate only.

Conclusions:- From a study of the figures in Table III it is evident that there is no relationship between agglutinin and complementary power. This is what one had been led to expect (Ritchie 1902 quoting Von Dungern).
If, however, during immunisation serumglobulin is increased and serum albumin diminished (Gibson and Banzhaf 1910), then it is very interesting that complement is not increased. For the theory that complement is a substance receives no support when increase of complement is not in line with increase of globulin during immunisation.

EFFECT OF AGAR ON COMPLEMENT.

Gardner (1917) described a method of obtaining a uniform yield of about 36% of serum from clotted blood, with no mechanical interference. The blood is collected in tubes lined with a thin film of agar (1.5% in 0.85% saline). This method Gardner found satisfactory in preparing agglutinating serum which suffered no loss in power. Incubation at 37°C speeded up coagulation which appeared to be slightly delayed in the agar lined tubes. He found gelatin lined tubes were also useful, but of course they could not be incubated at 37°C.

Teague and Buxton (1907) reported that agar destroyed complement, though they did not lay stress on this in an experiment on the electrical charges of complement.

Gardner's method of obtaining serum was thought possibly suitable for haemolytic work, so some experiments are arranged to test the effect of agar on complement.

Experiments:-/

Experiments:-

1. Guinea pig killed 27.12.20 and bled as equally as possible into three tubes. (A.) Ordinary sterile (B) Ordinary sloped nutrient agar. (C) Thinly lined with nutrient agar. Tubes were placed in ice chest for 24 hours, serum pipetted off from each tube and complement estimated. (A) M.H.D. 0.01. (B) and (C) No haemolysis in 0.015.

2. Guinea pig killed 20.1.21 and bled as equally as possible into three tubes. (A) Ordinary sterile. (B) Thinly lined with agar 1.5% in 0.85% saline. (C) Thinly lined with gelatin 10% in 0.85 saline. Tubes were placed in ice chest for 24 hours, serum pipetted off from each tube and complement estimated. (A) M.H.D. 0.005. (B) No trace of haemolysis in 0.55. (C) M.H.D. 0.0075. Then equal parts of serum A and B mixed and allowed to stand at room temperature for one hour. Then M.H.D. of A = 0.0075.

3. Fresh guinea pig serum (M.H.D. 0.005) was allowed to be in contact with agar 1.5% in 0.85% saline. After one hour at room temperature there was no appreciable/

appreciable diminution in complement. After being over night in ice chest there was no haemolysis with 0.55.

Conclusions:-

1. Gardner's method of obtaining serum in agar lined tubes cannot be used if the complementary activity of serum is wanted.

2. This method would be very suitable for taking specimens of human serum for the Wassermann test, as complement is destroyed and there is no anti-complementary activity of the serum.

3. Gelatin has practically no anticomplementary action.

GENERAL DISCUSSION.

Complement can be destroyed in various ways which afford an interesting study. A summary of work on this subject was given by Muir (1910).

Muir and Martin(1906) found that fixation of complement was as a rule closely associated with the formation of a precipitate.

Dean (1917) working with antigen and antiserum thought it probable that there is a direct relationship between the surface area of the particles of the precipitate and the amount of complement fixed. Also from his experiments "it can hardly be doubted that precipitation of the globulin of normal guinea pig serum is an essential part of the mechanism of complement fixation".

Scott (1911) showed that during anaphylactic illness complement is markedly weaker than before injection. As the animal recovers complement returns to its former strength usually within one or two hours even after severe symptoms.
Scott's work shows that destruction of complement in vivo is in line with destruction in vitro.

Wells (1918) argues that complement is a protein because it has antigenic properties so that immunisation/

immunisation with sera containing either complement or complementoid causes anticomplement activity in the blood of the immune animal. He also holds that the colloid nature of complement is attested by the large loss when it is filtered through Berkefeld filters. (Muir and Browning 1909).

All these experiments on the destruction of complement by no means prove that it is a substance. The theory that complement is a property of serum proteins can fit in with all these facts.

An interesting practical point has been observed in performing the Wassermann Reaction. In just over 200 bloods personally taken there were no anti-complementary specimens, i.e. no specimens of serum in the control (without antigen) fixed three or more doses of complement. Under similar circumstances in about 300 specimens taken by outside practitioners about 4% were found anticomplementary. These anti-complementary sera were almost certainly due to faulty technique in taking the specimen. Sera can readily become anticomplementary under various conditions, e.g. bacterial, thermal, chemical, mechanical (Browning and Mackenzie 1912). The remedy for trouble with such sera is apparently improved technique in taking specimens of blood.

P A R T II.

The following experiments on the nature of complement were carried out:-

1. Electrical conductivity.
2. Viscosity.
3. Freezing point.
4. Shaking.

1. ELECTRICAL CONDUCTIVITY.

Preliminary Considerations. Electrical conductivity of colloid solutions has been found to be exceedingly small. Whether the observed conductivity is not due to impurities is difficult to say. (Taylor 1919). Malfitano (1904) found that filtration through collodion of albumin and various colloids did not change the conductivity of the solution, though the collodion retained considerable quantities of the colloid. Now ionised substances may be produced from non-ionised. For instance proteins are split up by enzymes into peptones and amino-acids, substances which are ionised to a certain extent. The course of such a protein degradation may therefore be followed by observing the increase of conductivity or what is the same thing the decrease of resistance. (Philip 1913 (1)).

Similarly if destruction of complement is merely degradation of proteins, one would expect changes in conductivity corresponding to the amount of destruction of complement.

Experiments. Apparatus described by Philip 1913 (2) was employed. A small cell capable of dealing with 2 cc. of serum was used. The platinum electrodes were firmly fixed with an interval of about 2 mm. An intermittent current was passed through the cell containing the serum to be tested. By means of a revolving commutator, a resistance box, and a galvanometer it was possible to compare the resistances of two specimens of the same serum, (1) fresh, unheated (2) after being heated for ten minutes at 55°C. to destroy complement.

I am indebted to Dr W.W. Taylor for his help in the use of electrical apparatus which he set up.

Table IV shows the figures of the comparative resistances in ohms.

TABLE IV./

TABLE IV.

No.	Animal.	Age of serum in hours.	M.H.D.	Temperature at time of test.	Resistance of serum Unheated.	Heated.
1.	Rabbit (B)	22½	0.08	13.1°	32.8	32.5
2.	Guinea pig	24	?	"	33.0	32.3
3.	Rabbit (B)	"	0.06	15°	29.9	30.5
4.	Guinea pig	"	?	"	28.3	29.2
5.	Rabbit (B)	46½	0.06	13.25°	33	32
6.	" (W)	"	"	"	"	"
7.	Guinea pig	49½	less than 0.005	"	"	33
8.	"	"	"	"	34	"
9.	"	24	0.005	16°	32	31
10.	"	"	"	"	"	"

Conclusions:- From a study of Table IV it is evident that there is no relationship between complement and conductivity. The slight differences in the readings recorded are within the limits of experimental error.

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2. V I S C O S I T Y.

Preliminary Considerations. The viscosity of serum depends on many conditions such as temperature, concentration, protein content (especially globulin content - Chick (1914)), previous mechanical or thermal treatment, age, acidity or alkalinity, proportion and nature of electrolytes present. Any increase in the number of dissociated (electrically charged) colloid particles increases the viscosity. Heating serum for ten minutes at 55°C. also increases the viscosity. Now if destruction of complement is simply dissociation of protein, one would expect some relationship between the complement content of serum and the viscosity readings before and after the destruction of complement.

Experiments. Viscosity was tested by means of Scarpa's method as modified by Holker (1920). "The method consists in measuring the time t, which is occupied in drawing up through a vertical capillary tube sufficient liquid to fill a bulb at the top of the tube, and the time t_2 which this volume takes in flowing out from the bulb under the weight of the liquid. Under constant conditions/

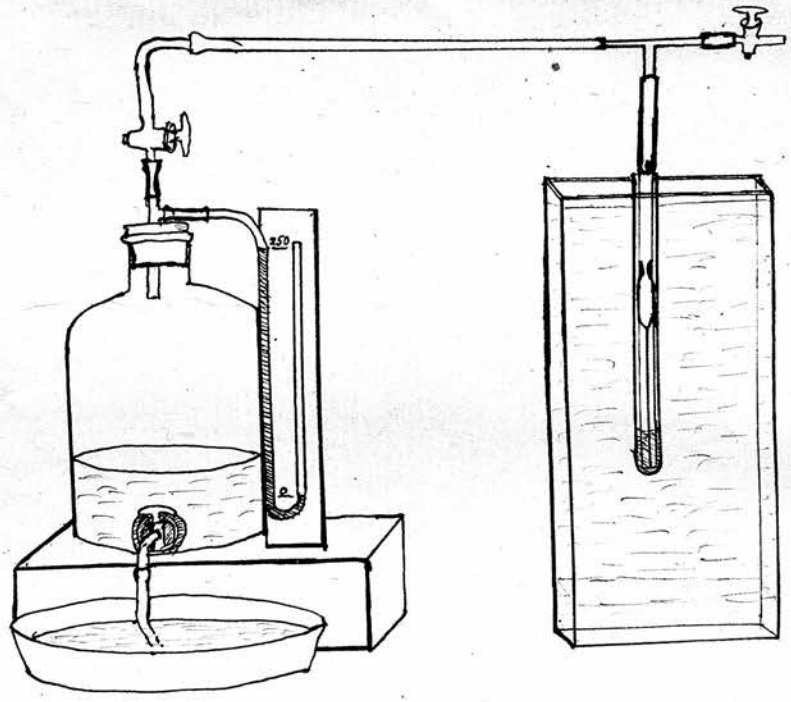

Diagram of Viscosity Apparatus

conditions the viscosity is proportional to the expression $\frac{t_1 \times t_2}{t_1 + t_2}$.

The capillary tube used in the experiments to be described had a bore of 0.5 mm. and a length of 13.0 cm. Above this was a bulb of about 1 cc. capacity. The tube was enclosed in a test tube 24 cm. long and 1 cm. wide, which was placed in a water bath maintained for the test at constant temperature. The negative pressure employed was equal to 250 mm. of water, and was accurately adjusted by varying the height of the "head" of water. The times were taken by a stop watch reading to $\frac{1}{5}$ second. Each reading was repeated at least three times and the average taken. The necessary data having been obtained, the viscosity relative to distilled water was calculated from the following formula:-

$$V = \frac{t_1 \times t_2}{t_1 + t_2} \times \frac{T_1 + T_2}{T_1 \times T_2}$$

where t_1 = time of upward flow of unknown

t_2 = " " downward " " "

T_1 = " " upward " " distilled water

T_2 = " " downward " " " "

TABLE V.

Source of Serum.	M.H.D.	V_1	V_2	Difference.
1. Guinea pig	0.01	1.23	1.28	0.05
2. Human	more than 0.8	1.23	1.26	0.03
3. Guinea pig	0.005	1.53	1.64	0.11
4. " "	0.01	1.51	1.62	0.11
5. " "	0.005	1.44	1.56	0.12
6. Horse	more than 0.5	2.33	2.45	0.12
7. Guinea pig	0.01	1.41	1.42	0.01

V_1 = Viscosity of unheated serum.

V_2 = Viscosity of serum heated 10 minutes at 55°C.

Conclusions:- From a study of Table V it appears that there is no obvious relationship between the amount of complement lost and the difference of viscosity. There is no evidence that complement is a substance destroyed by dissociation of protein.

3. FREEZING POINT.

Preliminary Considerations:- Raoult (1882) discovered that the lowering of the freezing point of a watery solution, as compared with the freezing point of distilled water, is proportional to the molecular concentration of the solution.

According to Arrhenius (1887) and van t'Hoff(1888) in partly dissociated solutions the ions have the same values as the undivided molecules in determining the lowering of the freezing point.

Reid (1904) showed that carefully purified albumin solutions had no osmotic effect and that the small osmotic pressure usually obtained is caused by foreign impurities, or by decomposition products of the albumin. For instance he found that metabolites of protein produced by bacterial growth will impart osmotic pressure to solutions of protein previously giving none.

On the other hand Lillie(1907) thinks it possible that Reid's low values for osmotic pressure of protein solutions were due to treatment with concentrated salt solutions to which the protein was subjected.

Experiments/

Experiments:- Freezing point was tested by means of Heidenhain's modification of Beckmann's apparatus. The thermometer was read to 0.002 degree by means of a lens. The zero was corrected with distilled water before each day's test. Serum had to be diluted owing to the quantity required. This was done immediately before the test by taking serum one part, distilled water four parts.
After the freezing point of unheated serum had been read, the thermometer was removed, and the serum in the freezing vessel was carefully heated to 55°C. and kept there for ten minutes. Care was taken to wash back into the serum any water condensed on the walls of the vessel. The freezing point of the heated serum was then taken.

TABLE VI.

	Source of serum.	M.H.D.	Depression of freezing point of serum diluted 1 in 5. A. Unheated.	B. Heated.	Difference.
1.	Guinea pig	0.01 ?	0.060	0.150	0.090
2.	" "	0.005	0.172	0.122	0.050
3.	" "	0.01	0.092	0.090	0.002
4.	" "	"	0.110	0.103	0.007
5.	" "	"	0.092	0.075	0.017
6.	Human	0.1	0.110	0.095	0.015
7.	" (Mixed)	"	0.135	0.127	0.008
8.	Guinea pig	0.0083	0.105	0.102	0.003
9.	" "	-	0.100	0.105	0.005

Note that only in Nos. 1 and 9 "B" is bigger than "A". In No.9 the serum was taken and mixed and then kept for two months in the ice chest until complement had disappeared. There was no trace of haemolysis even in 0.7 cc. of neat serum.
The guinea pigs were bled 24 hours before the serum was required for the test. The human serum was from the placenta.

Conclusions:- If there was disintegration of protein associated with destruction of complement one would expect a relationship between the depression of freezing point and the amount of complement destroyed. Table VI shows no such relationship.

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4. SHAKING.

Preliminary Considerations. Ramsden (1903) showed that mere agitation of various protein solutions brought about a separation of some of their contained protein in the form of fibrous or membrano-fibrous solids. He proved that it was possible in this way to coagulate and remove the whole of the protein from solutions of egg albumin.

Schmidt (1913-14) gives a summary of the literature on the subject of shaking serum. He found that albumins require to be shaken a longer time than globulins in order to be precipitated. He thinks shaking produces a progressing alteration of all the serum proteins leading finally to their denaturation. This alteration affects principally and first of all the euglobulin, and changes in the albumin and pseudoglobulin follow later, but always are present before visible coagulation takes place. While it is easy to destroy complement in fresh serum by shaking for a few hours at 37°C, prolonged shaking is necessary at temperatures below 20°C. before complement is perceptibly weakened. Sometimes fresh serum is practically unaffected by shaking at low temperatures (below 20°C.). Noguchi and Bronfenbrenner (1911) after shaking serum for ten hours at 10°C. found slight/

slight changes.

The following experiments were carried out in order to study the relationship of serum proteins and complement.

Experiments:- Specimens of guinea pig blood after 24 hours in ice chest were taken, and the serum was pipetted off and then centrifuged thoroughly. About 2 cc. of serum were placed in a stout old glass tube of 17 cc. capacity and 1.6 cm. diameter. The tube had a rubber stopper and was shaken horizontally in the dark at room temperature (8° - 13° C.). The shaker was driven by an electric motor and when working slowly made about 50 double excursions per minute, when fast about 100. As control part of the serum was kept close to the shaker but was not shaken. The turbid shaken serum was divided into two and one part was thoroughly centrifuged. The deposit which formed was amorphous and had no formed structure microscopically. It was light grey coloured, and was practically insoluble in serum, saline, distilled water or dilute acid. It cleared a little but not completely with dilute alkali.

TABLE VII.

No.	Hours since bled.	Time of shaking in hours.	M.H.D. Unshaken	M.H.D. Shaken & turbid	M.H.D. Shaken and then cleared by Centrifuging.
1.	144	1½	0.04	0.04	-
2.	72	"	"	"	-
3.	24	2¼	0.0125	0.0075	-
4.	48	6¼	0.0125	0.01	0.0125
5.	24	4¼	"	0.015	0.005
6.	"	2	"	0.0075	0.0075
7.	"	3¾	0.0075	"	"
8.	"	4	0.01	"	0.01
9.	"	"	0.0125	0.0125	0.0125
10.	"	5	0.01	0.01	0.0075
11.	"	"	"	"	"
12.	"	25	0.02	-	0.1
13.	"	10	0.01	-	0.015
14.	"	22	0.0075	-	0.01

Nos. 1 - 5 were shaken slowly with a small excursion.
Nos. 6 - 14 were shaken fast with a good excursion.

In all fourteen tests there was a distinct deposit formed by shaking. This varied from a slight haziness in No.1 to a considerable deposit in No.14. In No.14 by coagulating the serum (shaken and unshaken) and weighing the dried serum it was estimated that $\frac{1}{6}$ part (by weight) of protein had been removed as a deposit. The only test where there was a considerable reduction in complement in the shaken serum was No.12. In all the other tests there was either very slight reduction of complement, or even in some cases slight increase in power, too slight to be taken into account.

Conclusions:- From these experiments it is evident that it is possible to remove protein from serum without destroying complement. It seems unlikely that if complement is a protein it would remain behind in serum without being carried down in the deposit.

SUMMARY AND CONCLUSIONS.

Haemolytic complement was tested in healthy and diseased animals.

1. Complement can frequently survive at room temperature and in ice chest for three days unaltered in haemolytic activity.

2. Variations in complement in healthy animals are slight.

3. In acute and chronic disease there may be no change from normal complementary activity.

4. During immunisation complement does not vary with degree of immunity nor with agglutinin content of serum.

5. Complement is destroyed by agar, and is only slightly affected by gelatin.

6. Sera taken for Wassermann Reaction may through faulty technique readily become anticomplementary.

7. Experiments on electrical conductivity, viscosity, freezing point, and shaking of serum give no support to the view that complement is a substance. On the contrary it seems highly probable that complement is merely a property of serum.

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